THE EFFECT OF X-RAYS AND ULTRAVIOLET LIGHT ON THE UPTAKE *IN VITRO* OF [8-^{14}C]ADENINE AND [2-^{14}C]-PHENYLALANINE BY ISOLATED NUCLEI

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SUMMARY

1. The uptake of [8-^{14}C]adenine and [2-^{14}C]phenylalanine into isolated calf-thymus nuclei have been shown to be inhibited by X-irradiation.

2. X-irradiation of isolated calf-thymus nuclei inhibited the uptake of [^{14}C]-adenine into the nuclear RNA while the uptake into the DNA was unaffected.

3. X-irradiation of isolated rat liver nuclei and/or microsomes inhibited the uptake of [^{14}C]adenine by the nuclei. u.v. irradiation of the nuclei and/or the microsomes, in this system, gave rise to an inhibition of adenine uptake which was reversed by incubating in daylight.

4. X-irradiation of isolated rat-liver nuclei and/or mitochondria inhibited the uptake of [^{14}C]phenylalanine by the nuclei. u.v. irradiation of the nuclei and/or the microsomes, in this system, gave rise to an inhibition of phenylalanine uptake which was reversed by incubating in daylight.

INTRODUCTION

In the previous paper[1], it was found that the rates of uptake of [8-^{14}C]adenine and [2-^{14}C]phenylalanine by isolated rat liver nuclei were greatly enhanced by the addition of microsomes and mitochondria, respectively, to the incubation mixture. The present paper gives the results of some investigations on the effects of X-rays and ultra-violet light on these systems.

METHODS

Suspensions of rat liver nuclei, mitochondria and microsomes were prepared as previously described[1]. Nuclei were also prepared, as described by ALLFREY, MIRSKY AND OSAWA[2], from calf thymus. The calf thymus was obtained at the local slaughter house, from the animals as they were killed, and was packed in ice for transportation.

After preparation of the nuclear and cytoplasmic suspensions, 3 ml of the suspensions were transferred to petri dishes (3″ diameter). The suspensions were then irradiated as follows. The petri dishes were placed 10 cm from the X-ray tube and the contents stirred by means of a magnetic stirrer. The X-ray tube was a low tension

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References p. 154/155.

Machlett AEG60 tube with a beryllium window and working at 30 kV potential. A 1 mm aluminium filter was placed between the X-ray tube and the sample to be irradiated. The dose of X-rays given was varied by varying the time of irradiation, *e.g.* 900 R (45 sec), 300 R (15 sec), and 50 R (2.5 sec).

The suspensions were irradiated with u.v. light by placing the petri dish, containing the suspension, and the magnetic stirrer in a light-proof box. The u.v. source was a Mineralight (Model SL 2537a) fitted with a short wave filter. The light source was placed 15 cm from the petri dish. The energy output of the lamp was of the order of 1,100 ergs/mm^2/min since the total energy given out by the lamp was 8,000 ergs/mm^2/min and 86 % of the 253.7 mμ light was absorbed by the filter. The contents of the petri dish were stirred slowly throughout the irradiation period of 2 min.

Composite homogenates were prepared and incubated in the presence of 0.25 *M* sucrose–0.1 *M* sodium phosphate buffer (pH 7.3), 0.1 *M* glucose solution (containing 8.16 mg NaCl and 5.07 mg $MgCl_2 \cdot 6H_2O$ per ml) and either [8-^{14}C]adenine or [2-^{14}C]-phenylalanine in 25 ml conical flasks. The incubations were carried out at 37° with gentle shaking. At intervals throughout the incubation period, drops of the incubation mixture were transferred with a stirring rod to a glass microscope slide and smears made. The smears, when dry, were fixed, washed and autoradiographs set up as previously described[1].

In the experiments on the uptake of adenine by calf-thymus nuclei, some of the nuclear smears were treated with ribonuclease (RNase) or deoxyribonuclease (DNase) solutions, prior to setting up the autoradiographs. The fixed and dried nuclear smears were incubated in a bath of RNase solution (10 mg crystalline Armours RNase/100 ml distilled water) or DNase solution (1 mg Worthington DNase/100 ml distilled water). The incubations with the enzyme solutions were carried out at 37° for 2 h. At the end of the 2 h incubation, the slides were washed with 70 % ethanol for 20 min and then with 0.01 % non-radioactive adenine solution for 20 min. The slides were allowed to dry before preparing the autoradiographs.

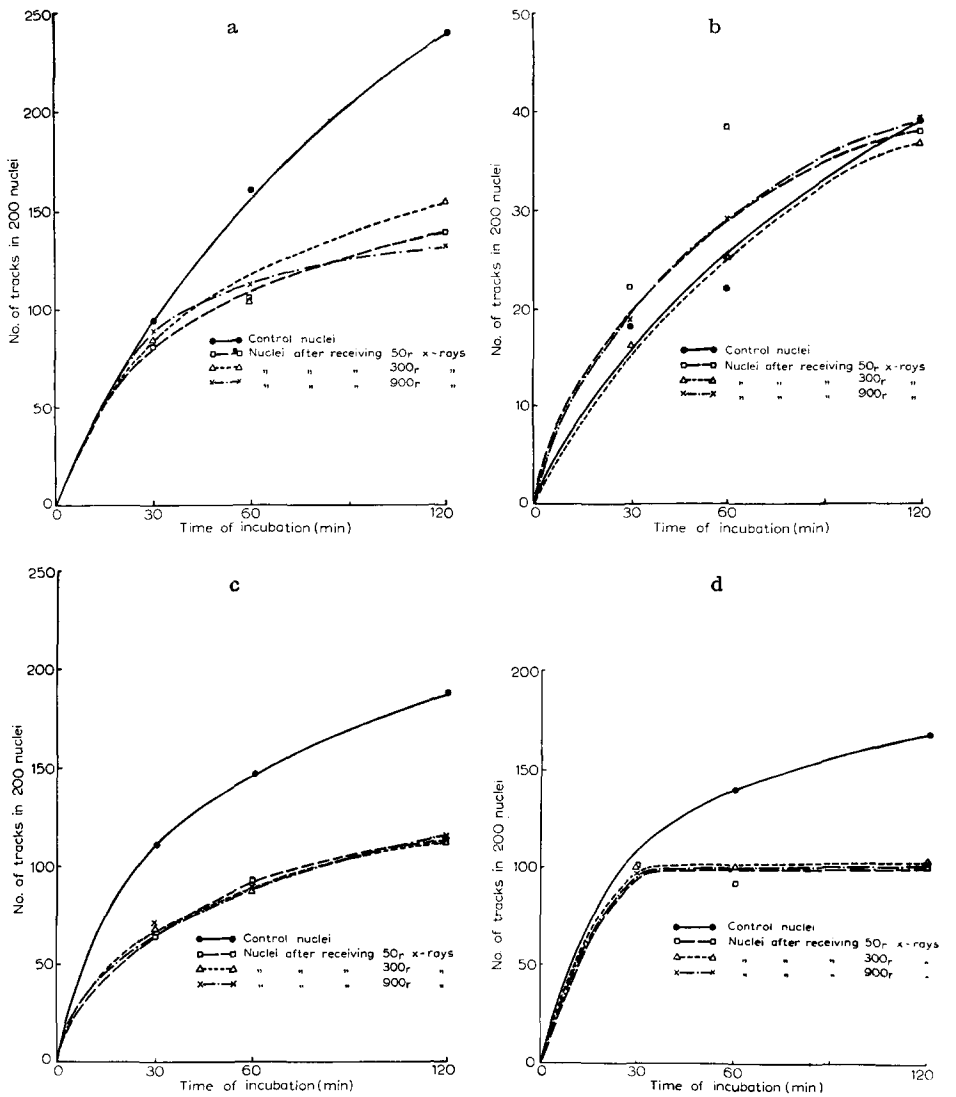
After leaving for 7 days in the dark, the photographic emulsion was developed, fixed and washed as previously described[1]. The nuclei were stained with methyl green-pyronin stain (Unna). The stained slides were mounted in balsam before examining under the microscope. 200 nuclei were examined, the number of nuclei which showed 0, 1, 2, 3, etc. tracks were counted and the total number of tracks in 200 nuclei determined.

RESULTS

The uptake of [8-^{14}C]adenine into the total nucleic acids of normal and X-irradiated calf-thymus nuclei is shown in Fig. 1a. The rate of uptake of the labelled adenine by the normal calf-thymus nuclei was rapid and was almost linear in relation to the time of incubation. Varying doses of X-rays were given to the irradiated nuclei, and it was found that the rates of uptake of the adenine by these nuclei were the same irrespective of the dose of X-rays given. X-irradiation of the nuclei, prior to incubation, inhibited the uptake of the adenine. During the first 30 min the uptake of adenine by the irradiated nuclei was approximately the same as that obtained in the control nuclei. After 60 and 120 min, however, there was a marked inhibition in the uptake of the labelled adenine by the irradiated nuclei. After 2 h incubation, all the irradiated nuclear fractions showed only about 60 % of the uptake shown by the control nuclei.

References p. 154/155.

Treatment of the nuclear smears with RNase, prior to autoradiography, removes the ribonucleic acid (RNA) from the nuclei, and consequently the activity observed was due to the deoxyribonucleic acid (DNA) and perhaps to some resistant RNA. The rates of uptake of the labelled adenine into the DNA of the control and irradiated nuclei are shown in Fig. 1b. In all cases the uptake of adenine by the DNA of the nuclei was approximately the same.



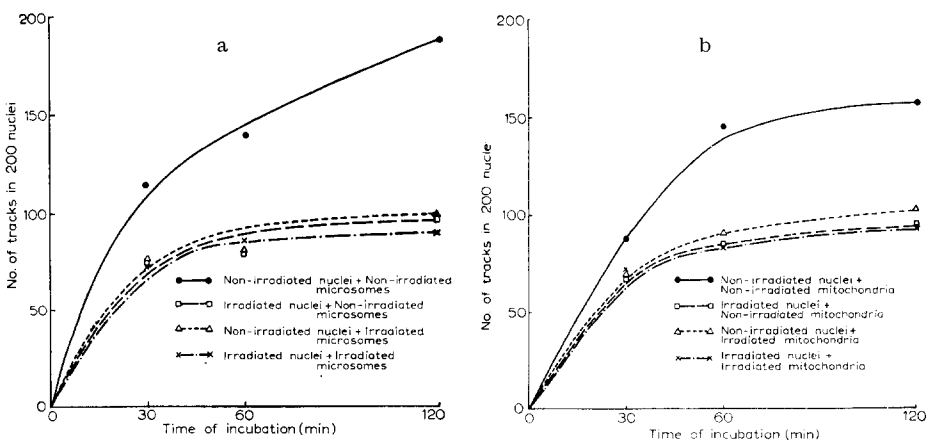
Figs. 1a, 1b, 1c and 1d. The uptake into normal and X-irradiated calf-thymus nuclei *in vitro* of [8-^{14}C]adenine into the total nucleic acids (1a), into the DNA (1b) and into the RNA (1c), and of [2-^{14}C]phenylalanine into the proteins. The incubation mixture contained 1 ml nuclear suspension, 0.5 ml 0.25 *M* sucrose–0.1 *M* sodium phosphate buffer (pH 7.3), 0.1 *M* glucose solution (containing 8.16 mg NaCl and 5.07 mg $MgCl_2 \cdot 6H_2O$ per ml) and 0.1 ml isotope solution (100 γ adenine $\equiv$ 2.5 μC, or 200 γ phenylalanine $\equiv$ 1 μC). The irradiated nuclei received 50 R, 300 R or 900 R of X-rays prior to incubation.

References p. 154/155.

When the nuclear smears were treated with DNase, prior to autoradiography, the DNA of the nuclei is removed and the observed activity is due to the adenine taken up by the nuclear RNA (Fig. 1c). The RNA of the control nuclei showed a rapid uptake of labelled adenine during the first 30 min of incubation. Between 30 and 120 min the rate of uptake by the control nuclei is almost linear, but at a slightly lower rate than that found during the first 30 min of incubation. In all the irradiated specimens there was a marked inhibition in the uptake of adenine into the nuclear RNA. With all doses of X-rays used, the degree of inhibition observed was the same. After 2 h incubation, the uptake of adenine into the RNA of the irradiated nuclei was approximately 60% that observed in the controls.

When the effect of X-irradiation on the uptake of [2-^{14}C]phenylalanine into the proteins was studied, using the same preparations of calf-thymus nuclei, the results shown in Fig. 1d were obtained. The control nuclei showed a rapid uptake of the labelled amino acid during the first 30 min. All levels of X-irradiation studied, gave rise to an inhibition in the uptake of the phenylalanine by the nuclei, and the degree of inhibition observed was the same. The uptake of the amino acid by the irradiated nuclei was approximately the same as that of the control nuclei during the first 30 min. In the subsequent 90 min, the control nuclei continued to take up the labelled amino acid, whereas the activity of the irradiated nuclei remained the same as that observed at 30 min. After 2 h incubation, the proteins of the irradiated nuclei showed only 60% of the activity shown by the proteins of the control nuclei.

In the previous paper[1], it was shown that the addition of microsomes to isolated rat-liver nuclei enhanced the uptake of radioactive adenine by the nuclei. The effect of exposing the nuclei and/or the microsomes to 300 R, prior to incubation in this system, is shown in Fig. 2a. It will be observed that X-irradiation of either the nuclei or the microsomes or both, inhibited the uptake of adenine by the nuclei throughout the incubation period.



Figs. 2a and 2b. The effect of X-irradiation on the uptake of [8-^{14}C]adenine (2a) and [2-^{14}C]phenylalanine (2b) by rat-liver nuclei *in vitro* in the presence of cytoplasmic components. The incubation mixture consisted of 0.5 ml nuclear suspension, 0.5 ml suspension of cytoplasmic fraction, 0.5 ml 0.25 *M* sucrose–0.1 *M* sodium phosphate buffer (pH 7.3), 0.4 ml 0.1 *M* glucose solution (containing 8.16 mg NaCl and 5.07 mg $MgCl_2 \cdot 6H_2O$ per ml) and 0.1 ml isotope solution (100 γ adenine $\equiv$ 1 μC or 200 γ phenylalanine $\equiv$ 1 μC). The irradiated specimens received 300 R of X-rays prior to incubation.

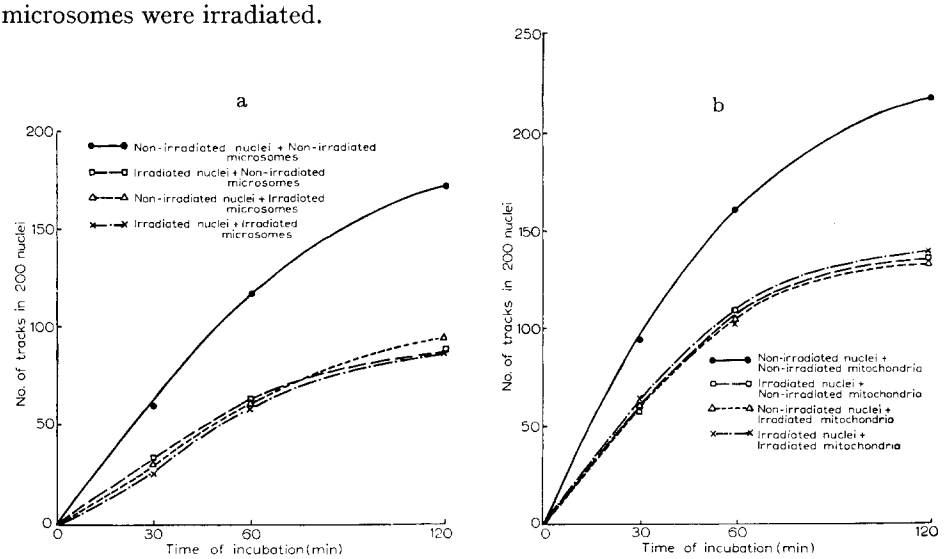
References p. 154/155.

The system, containing the irradiated fractions showed an uptake of the labelled adenine during the first 30 min, after which there was little or no further uptake of the adenine by the nuclei. The degree of inhibition obtained was the same, irrespective of whether it was the nuclear fraction or the microsomal fraction or both fractions which had been irradiated. In every case, the inhibition in the uptake of adenine by the nuclei, after 2 h incubation, was approximately 50 %.

It was also shown in the preceding paper[1] that the addition of mitochondria to an incubation mixture containing isolated rat-liver nuclei enhanced the uptake of [2-^{14}C]phenylalanine by the nuclear proteins. Fig. 2b shows the effect of 300 R on the nuclei and/or the mitochondria in this system. The irradiation of either the nuclei and/or the mitochondria, prior to incubation with the labelled amino acid, gave rise to the same degree of inhibition in uptake of the labelled phenylalanine by the nuclei. This inhibition was marked throughout the period of incubation studied and after 2 h amounted to about 33 % in all cases.

A similar study was made on the effect of u.v. irradiation on these systems. Two series of experiments were carried out on the u.v.-irradiated systems. In the first series of experiments, all the manipulations and incubations of the irradiated systems were carried out in the dark; in the second series of experiments, no precautions were taken to exclude light while working with or incubating the irradiated material.

Fig. 3a shows the results obtained when the nuclei and/or the microsomes received 2,200 ergs/mm^2 of short wave u.v. irradiation, prior to incubation in the dark with labelled adenine. It will be noted again that there was a marked inhibition in the uptake of adenine by the nuclei, irrespective of whether the nuclei and/or the microsomes were irradiated.



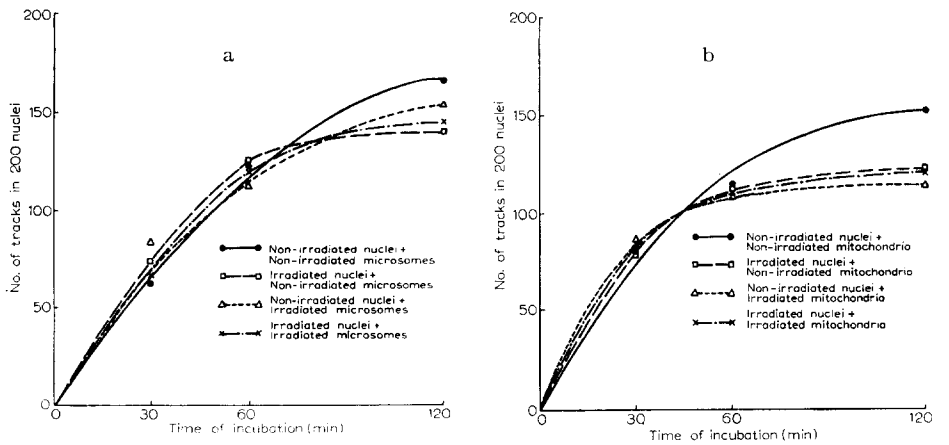
Figs. 3a and 3b. The effect of u.v. irradiation on the uptake of [8-^{14}C]adenine (3a) and [2-^{14}C]-phenylalanine (3b) by rat-liver nuclei *in vitro* in the presence of cytoplasmic components. The incubation mixture consisted of 0.5 ml nuclear suspension, 0.5 ml suspension of cytoplasmic fraction, 0.5 ml 0.25 *M* sucrose–0.1 *M* sodium phosphate buffer (pH 7.3), 0.4 ml 0.1 *M* glucose solution (containing 8.16 mg NaCl 5.07 mg $MgCl_2 \cdot 6H_2O$ per ml) and 0.1 ml isotope solution (100 γ adenine $\equiv$ 1 μC or 200 γ phenylalanine $\equiv$ 1 μC). The irradiated specimens received 2,200 ergs/mm^2 of u.v. light prior to incubation and all the manipulations and incubations were carried out in the dark.

References p. 154/155.

An inhibition in the uptake of labelled phenylalanine by the isolated nuclei was also noted when either the nuclei and/or the mitochondria were irradiated with u.v. and the incubation carried out in the dark (Fig. 3b). Again the same degree of inhibition was observed, regardless of whether one or both of the cellular fractions were irradiated.

When these experiments, on the effects of u.v. irradiation, were repeated under identical conditions, except that the incubations were carried out in the open laboratory and no precautions were taken to exclude day light, the results, shown in Figs. 4a and 4b, were obtained. Fig. 4a shows the uptake of adenine by the nuclei under these conditions. It will be seen that no inhibition was apparent until after 2 h incubation, and even then the degree of inhibition obtained was very small.

The effect of incubating nuclei and mitochondria with labelled phenylalanine under these conditions is shown in Fig. 4b. It will be noted that the degree of inhibition obtained was much less than that observed when the specimens were incubated in the dark (Fig. 3b). Compared with the uptake of labelled adenine under the same conditions, visible light did not appear to reverse to the same extent the inhibition in the uptake of phenylalanine produced by u.v. light. The nuclei, in all the irradiated systems, showed no inhibition in the uptake of labelled amino acid during the first 60 min, but after 2 h, inhibition amounting to 20 % was observed.



Figs. 4a and 4b. The effect of u.v. irradiation on the uptake of [8-^{14}C]adenine (4a) and [2-^{14}C]-phenylalanine (4b) by rat-liver nuclei *in vitro* in the presence of cytoplasmic components. The incubation mixture consisted of 0.5 ml nuclear suspension, 0.5 ml suspension of cytoplasmic fraction, 0.5 ml 0.25 *M* sucrose–0.1 *M* sodium phosphate buffer (pH 7.3), 0.44 ml 0.1 *M* glucose solution (containing 8.16 mg NaCl and 5.07 mg $MgCl_2 \cdot 6H_2O$ per ml) and 0.1 ml isotope solution (100 γ adenine $\equiv$ 1 μC or 200 γ phenylalanine $\equiv$ 1 μC). The irradiated specimens received 2,200 ergs/mm^2 of u.v. light and no precautions were taken to exclude daylight during the manipulations or incubation.

DISCUSSION

It is generally found when RNA is treated with RNase that about 60 % of the RNA is hydrolysed by the enzyme and that there is a RNase-resistant "core" left. If this is the case, the activities shown in Fig. 1b were due to this RNase-resistant "core" + DNA. However, when the nuclear smears were treated with RNase followed by DNase

References p. 154/155.

treatment, the activity observed was of the same order as the normal background (2–4 tracks/200 nuclei). (The nuclear smears, after RNase + DNase treatment, were stained with mercuric bromophenyl blue[3], prior to setting up the autoradiographs.) It was also found that the sum of the activities after RNase treatment (Fig. 1b) and the activities after DNase treatment (Fig. 1c) was almost the same as the total activities observed (Fig. 1a). It would appear, therefore, that either the treatment with RNase removed all of the nuclear RNA, or that the RNase-resistant RNA contained little or no labelled adenine. Of these two possibilities, the second is the more likely.

It has been shown by many workers, among them HEVESY[4], VERMUND, BARNUM, HUSEBY AND STENSTOM[5], and HOLMES AND MEE[6], that DNA formation in growing tissues can be reduced to 50 % of normal by X-irradiation. VAN EULER AND HEVESY[7] have shown that the uptake of precursors into DNA can occur in two ways, (a) during the formation of new DNA, which occurs during mitosis, and (b) during the turnover of the DNA already present. Of these two processes only (a) is radiosensitive. In the present work, it is possible that the uptake of adenine observed (Fig. 1b), is due to turnover of the DNA. As for the control calf-thymus nuclei, it is possible that the isolation procedure has inhibited the formation of new DNA, and again the uptake of adenine observed is due to turnover of the DNA already present. This would account for no difference being found between the control and the irradiated nuclei.

It has been found by many workers[4–6] that when whole cells are irradiated, there is little or no effect on RNA synthesis. It would appear from the present investigations on isolated calf-thymus nuclei (Figs. 1a, 1b and 1c)) that X-irradiation of the nuclei, prior to incubation with [^{14}C]adenine, inhibits the uptake of adenine into the nuclear RNA. Since the degree of inhibition observed was the same, irrespective of the dose of X-rays given, it would suggest that as little as 50 R of X-rays were sufficient to inhibit the uptake of adenine by the nuclear RNA. It is possible that the inhibition may be due to the formation of peroxides in the sucrose medium. In this respect 50 R is a very low dose of X-rays; normally the dose of radiation supposed to give sufficient peroxide formation is at least 100 times greater. Secondly, it must also be supposed that 900 R would give rise to a much greater formation of peroxides, and therefore the degree of inhibition observed would have been proportionally greater. In a recent series of experiments LOGAN[8] has found that irradiation of the sucrose medium has no effect on the uptake of labelled precursors into non-irradiated nuclei.

Using the same nuclear preparations, there was complete inhibition in the uptake of labelled phenylalanine by the X-irradiated nuclei after the initial 30-min incubation (Fig. 1d). In the case of adenine uptake into the nuclear RNA, there was a marked but not complete inhibition in the rate of uptake throughout the incubation period.

GALE AND FOLKES[9] have shown that in staphylococci, the synthesis of protein and RNA occur simultaneously, but protein synthesis can be blocked by antibiotics without RNA synthesis being affected[10]. It would appear, therefore, that in isolated calf-thymus nuclei X-irradiation can block protein synthesis completely while RNA synthesis is only partially reduced. DNA synthesis, on the other hand, would appear to be unaffected.

From the results obtained when rat-liver nuclei and/or rat-liver microsomes were exposed to X-rays (Fig. 2a) or u.v. light and incubated in the dark (Fig. 3a), the uptake of adenine was markedly inhibited. This suggests that (a) both the nuclear and microsomal systems are involved in the mechanisms responsible for the adenine being

References p. 154/155.

incorporated in the nuclear nucleic acids, or (b) the irradiation of the sucrose medium gives rise to toxic compounds which block the uptake of the adenine by the nuclei. Of these two possibilities the first would seem to be the most likely, since LOGAN[8] has been able to show that irradiation of the sucrose medium does not affect the uptake of labelled precursors into non-irradiated nuclei. Also, when the cellular particles were irradiated with u.v. and the incubation was carried out in daylight (Fig. 4a), little or no inhibition was observed. If any toxic compounds had been formed by irradiation of the sucrose, it is unlikely that visible light would remove these compounds or convert them to other less toxic compounds.

It has been shown in the previous paper[1], that the nuclei alone are capable of taking up free adenine. This would therefore suggest that in the presence of irradiated microsomes there is no free adenine available for the nuclei to take up. It is possible that the adenine is taken up by the irradiated microsomes where it is bound and can no longer be transferred to the nuclei.

It would therefore appear that both the nuclear and microsomal systems, concerned in the nuclear uptake of [^{14}C]adenine from the incubation medium, are sensitive to X- and u.v. irradiation. Visible light would also appear to overcome the inhibition arising u.v. irradiation. In the case of [^{14}C]phenylalanine uptake by the nuclei in the presence of mitochondria, it would seem that both the nuclear and mitochondrial systems are necessary.

VAN BEKKUM[11] has reported some studies on isolated spleen mitochondria after irradiation. He found that, even with doses of X-rays as high as 20,000 R, oxidative phosphorylation was unaffected. FRITZ-NIGGLI[12,13] disagrees with these findings since she found that her preparations of rat-liver mitochondria were sensitive to small amounts of X-irradiation, the enzymes of the citric acid cycle being inhibited by 50 R and oxygen uptake by as little as 0.1 R of X-irradiation. This would seem to indicate that the effect we observed was due to a block in the formation of active phosphate compounds. However, it is possible that the enzymes concerned in the "activation" of the phenylalanine are also inhibited by irradiation.

OTTOLENGHI, BERNHEIM AND WILBUR[14] have shown that u.v. irradiation of isolated rat-liver mitochondria inhibits succinoxidase activity. They also showed that u.v.-irradiated or oxidised lipids inhibited succinoxidase activity. This inhibition could be partially reversed by the addition of glutathione to the medium. In the present work it was found that the inhibition obtained with u.v. irradiation could be almost completely reversed by incubating in daylight. It is possible that the photoreactivation observed was due to the formation of reducing compounds.

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STUDIES ON HYALURONIC ACID

I. THE INFLUENCE OF IONIC STRENGTH ON THE SEDIMENTATION AND DIFFUSION PROPERTIES*

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SUMMARY

Hyaluronic acid was prepared by an electrophoretic separation technique. This was considered to be the mildest possible method of preparation and the method least likely to cause any changes during preparation.

The following physicochemical parameters were obtained: Sedimentation constant, $s_{20} = 3.1$ S; diffusion constant, $D_{20} = 1.25 \cdot 10^{-7}$ cm²/sec; intrinsic viscosity, $[\eta] = 7.0$. From these values we were able to calculate a molecular weight of M = 178,000 and a hydrodynamically effective volume of $V_e = 1.0$ ml/g, indicating a hydration of about 30 %. On this basis and on the assumption that the shape of the molecule approximates the shape of a prolate ellipsoid of revolution, the molecular length was calculated to be $L = 1900$ Å and the diameter of maximal circumference, $d = 17.5$ Å.

A frequency distribution coefficient of $\gamma = 0.44$ was calculated from the spread of the sedimentation patterns. It was found that hyaluronic acid satisfies the Huggins equation when the constant $k' = 0.275$.

The sedimentation and diffusion characteristics of hyaluronic acid have been studied as a function of ionic strength. It was found that these properties are very sensitive to changes in ionic strength, with the dependence most marked as zero ionic strength is approached. However, the changes induced by varying the ionic strength are perfectly reversible. Changes in temperature had no effect within the range investigated.

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References p. 165.